THE UNIVERSITY OF ALBERTA

EFFECTS OF REPELLENTS ON MOSQUITO BEHAVIOUR

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ENTOMOLOGY

EDMONTON, ALBERTA

SEPTEMBER 1963

ABSTRACT

The behaviour of Aedes aegypti L. and other species of Aedes in relation to repellent chemicals was studied. The repellents used were dimethyl phthalate, ethyl hexanediol, N,N-diethyl metatoluamide and indalone. The effect of these repellents on the behaviour of mosquitoes was studied in two ways: firstly by placing the repellents on selected parts of the environment of mosquitoes and secondly by painting them on parts of mosquitoes themselves where chemoreceptors are known to occur, such as the antennae, labium, and tarsi. The aspects of behaviour studied were, feeding on blood and on sugars, mating, oviposition, the reactions to wind, geotaxis, and orientation to centrifugal force, and the visual response to black stripes. All these aspects of behaviour are affected significantly by repellents. Dimethyl phthalate has the greatest effect of the four repellents on blood feeding behaviour when they are painted on the tarsal receptors and the smallest effect when they are painted on the receptors of the antennae and the labium.

The experiments provided some understanding of the mode of action of insect repellents. They suggest that repellents interfere with normal behaviour perhaps by blocking the olfactory receptors mediating both attraction to the host and that to raisins, the contact chemoreceptors invoking feeding on blood and those used in the selection of oviposition sites. The experiments also show that mechanoreceptors effecting orientation to gravity and air flow and visual receptors effecting orientation to black stripes are also perhaps interfered with by repellents. There is also some evidence that repellents block the thermoreceptors which may mediate piercing for feeding on blood and perhaps auditory organs involved in mating. The only receptors which the repellents do not appear to interfere with seem to be those of the common chemical sense.

ACKNOWLEDGEMENTS

I would like to thank with a feeling of deep gratitude Prof. B. Hocking for his many valuable suggestions and keen interest in the work, and for his readiness to discuss any problem arising during the course of this study; Dr. W.G. Evans for his many helpful suggestions; Dr. G.E. Ball for his encouragement and help in many ways during my stay in Canada; and Dr. J. Sharplin for her interest in the work. I also want to thank Dr. K.M. Chapman and Dr. C.P. Hickman, members of my committee, for many useful suggestions.

My thanks are also due to Miss J.C. Shore, and Miss A Zalums for their co-operation and help; and Mr. E. Newman of the Works Department, University of Alberta, for the loan of an anemometer. I would also like to thank Mr. Malcolm J. Reddy, a fellow graduate student for helping me in some of the experiments.

I gratefully acknowledge the support of the Defence Research Board, Department of National Defence, Government of Canada, who financed this study. My special thanks are due to the Council of Scientific and Industrial Research, Pakistan, who paid my passage to Canada and treated me as on duty during my stay in this country.

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1. INTRODUCTION

From very early in his history man has been most concerned with simple and quick methods of dissuading insects from inhabiting his dwellings, his clothing, and his person. Yet no repellents were marketed until the end of the nineteenth century. The bulk of research on repellents has been defence orientated, mainly by the requirements of the second World War. From about 1901 oil of citronella was the most widely used repellent and was the repellent against which new compounds were tested. At the start of World War II, dimethyl phthalate, indalone and Rutger's 612 were mixed and the mixture designated as 6-2-2 was recommended to be used as a standard repellent against blood sucking arthropods, particularly mosquitoes. This formulation did not quite give the protection demanded by military requirements and thus provided the stimulus for increased and accelerated research on repellents.

The discovery of the transmission of malarial parasites by Ross (1898) and the discovery by Walter Reed and his collaborators that yellow fever was transmitted by Aedes aegypti led to the realization of the importance of mosquitoes as carriers of disease. Repellents being a cheap and efficient means of individual protection against these insects were studied by many workers in an attempt, firstly to develop new and better repellents and secondly to understand the mode of action of these compounds.

Aedes aegypti L. was used for most of the following study on repellents. The mosquitoes were reared in the laboratory at 80 $\pm$ 2°F. and at an R.H. of 60% $\pm$ 5. Breeding females were fed on blood from the arm. The suggestions made by Kalmus and Hocking (1960) were followed up, and the behaviour of Aedes aegypti towards repellents was studied not

only in relation to blood feeding but also in relation to sugar feeding, mating, oviposition, geotaxis, wind direction and speeds, and visual responses to black stripes. The repellents used were: dimethyl phthalate indalone, diethyl toluamide, and Rutger's 612. The first two are esters, the third an amide and the last named an alcohol. These are compounds of low volatility and moderate molecular weight. They are insoluble or only very slightly soluble in water but are miscible with alcohols and ether. Their physical and chemical properties are listed in Table 1.

TABLE 1

Chemical and physical properties of the repellents used in the study of behaviour of Aedes

Common Name	Chemical Name	Mol. Wt.	b.p.	Solubility and Miscibility
Dimethyl phthalate	dimethyl benzene-ortho-dicarboxylate	194.18	285°C	0.43% w/w soluble in water
Indalone	n-butyl mesityl-oxide oxalate	226.26	113°C	Insoluble in water; miscible with alcohol
Diethyl toluamide	N,N-diethyl m-toluamide	191	111°C at 1 mm. pressure	Insoluble in water; miscible with alcohol
Rutger's 612	2-ethyl hexane-diol-1,3	146.22	244°C	Slightly soluble in water; miscible with alcohol

2. REVIEW OF LITERATURE

2.1 Sense Organs of *Aedes aegypti* L.

2.11 Introduction

An *Aedes aegypti* female is attracted to its host in part through the chemoreceptors located on various head appendages but mainly on the antennae. Bishop and Gilchrist (1946) showed that in *A. aegypti* eyes are not essential for feeding on blood. They demonstrated that *aegypti* can feed on the human arm in complete darkness and as far as they could ascertain, with no delay in settling or gorging. Roth (1951 p. 60) also reported that eyes are not necessary in locating the host in a small area such as a cage.

DeLong (1946) considers antennae to function as directional receptors, possibly receiving the stimuli which induce the probing response. He considers antennae and the palps the chief organs for locating the host and stimulating probing. According to him the antennae may perform both functions but the palps can receive stimuli only when the insect is directly on the skin. Roth (1951) also considers the antennae and the palpi as the chief organs used by *Aedes aegypti* females in locating the host and receiving the stimuli which induce probing. According to him the antennae function as directional thermoreceptors and probably chemoreceptors as well. Like DeLong, he also subscribes to the view that the palpi receive stimuli when the insect is on, or near, the skin of the host. Roth also reported temperature receptors on the palps of *A. aegypti*. Dethier (1952) thinks that receptors on antennae, mouth parts & tarsi function at different levels of sensitivity. The antennae according to him are the most sensitive and the various mouth parts less so. Rahm (1958) reiterates the view expressed above and concludes that antennal amputation shows that these organs are essential for host finding and attraction from

a distance. He also reports that antenna-less mosquitoes can probe and suck if the palps remain intact. A brief description of these chemoreceptors is given below under their site of location.

2.12 Antennae

The antennae in male and female aegypti consist of a basal ring-like scape, a globular pedicel, and a long flagellum of thirteen articles. The pedicel in both sexes contains Johnston's organ, which is more developed in the male. In the flagellum of the female aegypti the second article is the shortest the remaining articles longer, the thirteenth being one tenth as long as the whole flagellum. Each article consists of a basal ring and a membranous area. Arranged around the article and borne on the membranous area is a whorl of five or six hairs.

Roth and Willis (1952) reported that many thin walled trichoid sensilla are present on each of the thirteen flagellar articles of the female aegypti and on the two terminal flagellar articles of the male. The results of their experiments led them to conclude that these serve as hygroreceptors.

Christophers (1960, p. 663) describes the trichoid sensilla of Aedes aegypti as "...40-50 u in length, thin walled and without articulated base, arising from thin membrane over a pore canal surrounded distally by a semicircular ridge in the cuticle. Proximally a median ridge passes backwards. They are set at regular intervals along the length of the segment and in the female number some 30-40 on a segment."

Steward and Atwood (1963) identified five structural types of sensilla on the antenna of the female aegypti. Three of these types they found thin walled and classified them as Ai, A2, and A3. According to them a typical A1 sensillum is 0.06 mm. long, curved and tapering to a

sharp point. Type A2 is shorter, 0.04 mm. long and with a blunt tip. Both are about the same diameter. Sensilla of A2 type are more closely appressed to the antennal shaft than those of type A1. In both types the sensillum is set in a shallow socket to which it is attached by a thin pigmented membrane. A sclerotized spine projects backwards from the base of the sensillum and adheres somewhat loosely to the cuticle on which it lies. This spine confers rigidity on the sensillum. The innervation of the two types is essentially the same. A group of 5 or 6 nerve cells lies posterior to the setal socket under the cuticle. A nerve fibre extends into the whole length of the setal lumen. This nerve fibre is made up of nerve processes from at least three nerve cells. Steward and Atwood described type A3 as a short, curved, thin-walled peg organ which is innervated by a group of nerve cells. Sensilla of type A1 and A2 were found more numerous on the distal articles and their number was found to decrease regularly towards the proximal end. Sensilla of type A3 were found to be located chiefly on the proximal articles of the antennal flagellum decreasing in number towards the distal end. Steward and Atwood also conclude from experimental evidence that types A1 and perhaps A3 play a major role in mediating attractancy while type A2 are responsible for mediating repellency.

Slifer and Sekhon (1962) studied with both the light and the electron microscope, the fine structure of the sense organs on the antennal flagellum of Aedes aegypti and found that five types of sensory hairs could be distinguished on the flagellum. All five are present in both the male and the female but the number of each and their distribution on the antennae differ markedly between the two sexes. No coeloconic or ampullaceous sense organs were found. The heavy walled hairs could be separated into those which are arranged in whorls and those not so arranged. The

former have a greater average length but the size limits of the two types overlap. Basically, these hairs resemble hairs of various sizes found elsewhere on the body and appear to be mechanoreceptors. According to the authors: "The thin walled hairs with a sharp tip have a complex wall. There is some evidence that the dendrites in the lumen reach the surface through twisted canals, and end at extremely small openings in the surface of the hair. It is probable that these structures serve as chemoreceptors." The thin walled hairs with blunt tips have been described as having many small openings in their walls. The lumina of these hairs contain dendrites which branch as they reach the distal end of the hair. The tips of the dendrites most probably lie in these small pores and these hairs are believed to be olfactory in function. The authors could not identify in electron micrographs with certainty the third type of these thin walled hairs which are small and thorn shaped in structure.

2.13 Palpi

Roth and Willis (1952) have described the female palps of *Aedes aegypti* as abundantly supplied with thin-walled club-shaped sensilla on the terminal segment. On the ventral side where the sensilla are mainly present, the segment is free from scales. These club-shaped hairs which are 20-30 u in length arise from a membrane covering a circular pore canal surrounded by a circular ridge. Pointed trichoid sensilla are also present. There is also a central short sclerotized peg at the extreme tip of the palp.

2.14 Labium

Frings and Hamrum (1950) have described the sensory structures on the labella of *Aedes aegypti*. They note four kinds of hairs in both sexes. (1) Non-sensory epicuticular hairs about 7 u long. (2) Hairs about 40 u

long lying at the tip of the labella in a fan shaped manner. These are considered to be tactile in function. (3) Curved hairs about 20 u in length at the tip and on the ventral surface, which may be chemoreceptors, and (4) About 14 hairs 6 u in length, of unknown function, on the dorsal surface of each labellum.

2.15 Tarsi

On the tarsi of the fore and mid legs of A. aegypti there are many slightly curved hairs probably tactile in function (Frings and Hamrum, 1950). Frings and Hamrum also note the presence of groups of short curved hairs similar to those found on the labella. These are considered to be tactile in function. Wallis (1954) found in Aedes aegypti that all tarsal segments were provided with curved thin walled spines corresponding in distribution with the sensory areas. Slifer (1962) describes the hairs on the tarsi as approximately 100 in number in the female. These hairs stain at the tip when dye is applied to the external surface of the insect. The male has about 60 of these hairs. In both sexes she finds the largest number occurring on the prothoracic tarsi and the smallest number on the metathoracic. None were found elsewhere on the legs. She concludes: "Little doubt now remains that the hairs with stainable tips are the tarsal gustatory receptors of the mosquito."

2.2 Mode of Action of Olfactory Receptors

Several theories have been advanced by different workers to explain the mode of action of olfactory receptors. Jones and Jones (1953) reviewed the modern theories on olfaction and classified them into the following categories:

a) Mechanical theories in which the stimulus is conceived of as a mechanical force.

b) Radiation and vibration theories in which either the stimulus or the qualitative differences are thought to be in some way dependent upon the molecular vibrations.

c) Stimulus pattern theory in which qualities of odor are determined by patterns of stimulations.

d) Chemical theories in which some sort of effect best described as chemical in nature is considered the stimulus.

e) Steric theory in which characteristics of odorous substances are ascribed to the shape and dimensions of the molecules.

f) Phase boundary theories in which an effect occurring at some sort of phase boundary is the stimulus.

Davies (1962) proposed that the mechanism of olfaction is the penetration and dislocation of a small region of the wall of an olfactory nerve cell. This dislocation allows the K and Na ions to move across the membrane, so initiating the nerve impulse. The effects of odorants on red blood cells as weak hemolysants and calculations of olfactory thresholds, are both in agreement with this penetration theory.

Amoore (1963) favours the stereochemical theory of olfaction. He says: "The odor of a chemical is in fact determined by the structure of the molecule, in particular by its size and shape. Inspection of the stereochemical formulae of more than six hundred chemicals has shown that without an exception they conform to a spherical shape and a size of 7 Å diameter within remarkably narrow limits. Hence it may be supposed that the olfactory epithelium contains 'receptor sites' to which it now becomes possible to ascribe definite shapes and dimensions. If a chemical is volatile, and its molecules have the appropriate configurations to fit closely into the receptor site, then a nervous impulse will be initiated ,

possibly through a mechanism involving disorientation and hence depolarization of the receptor cell membrane." Amoore was able to foretell on the basis of this theory the odor of several compounds before they were synthesized.

2.3 Factors Responsible for Attracting Mosquitoes to the Host

The mode of action of insect repellents cannot be properly studied without an understanding of the factors that attract the insect to the host, in this study the female *Aedes* mosquitoes to man. Contradictory views can be found in the literature on this point; all workers accept the physical factors, temperature and humidity, as attractants; others consider chemical stimuli, carbon dioxide and host odor, or only carbon dioxide to be also important in attracting the mosquito to its host.

Howlett (1910) believed temperature to be the chief attractant and said that the smell of sweat or of blood was not attractive. Reuter (1936) showed that moisture was distinctly attractive to *A. aegypti*. Van Thiel (1937) assigned the role of attraction chiefly to the physical factors of temperature and humidity and the chemical factor of carbon dioxide. He thought that in comparison with these stimuli, almost no attraction was exercised by the odor of human or animal blood, but on further study Van Thiel and Laarman (1953) consider that the scent of the host plays an important part in the orientation of the mosquito toward it, and went to the extent of saying that it could be assumed that the mosquito can react by means of directed movement to the prey, even at a distance where the heat, humidity, and carbon dioxide of the blood supplier had no influence.

DeLong, Davidson, Peffly and Venard (1945) found moistened warm air more attractive to *A. aegypti* than warm air. Most of these tests were

conducted with olfactometers or inanimate objects. Brown (1958) in tests with human subjects observed that the hands of individuals with low moisture output attracted more mosquitoes than those with high output, and that warm skin was more attractive than cool. According to Brown (1956): "The realization that adult female mosquitoes approach their animal host not as an act of volition, but as a token response to certain stimuli that the host introduces into the environment, has prompted the analytical method of studying the individual factors that might constitute stimuli." He then goes on to recognize six factors which guide the female mosquitoes to their animal hosts, three of these being air-borne (water vapour, carbon dioxide, and convective heat) and three visual (movement, contour, and reflectivity). He also says that the search for the attractive elements in sweat other than CO_2 proved fruitless and that carbon dioxide is responsible for only a certain fraction - about a quarter - of the attractiveness of beef blood to A. aegypti in the laboratory. Attractive elements in the blood other than carbon dioxide remain unknown.

Kellogg and Wright (1957) and Wright (1962) consider moisture and carbon dioxide to be the main attractant factors. Christophers (1960, p. 535) remarks: "On the whole the evidence that smell is an important stimulus in the attraction of A. aegypti to feed is not very strong."

On the other hand many say that body odor plays an important role in the attraction of mosquitoes. Goeldi (1905) reported perspiration to be the agent attracting mosquitoes to man. Haddow (1942) reports that an unwashed African child attracted more Anopheles gambiae Giles, Anopheles pharoensis Theobald, and Anopheles funestus Giles than a clean child, and huts containing dirty clothes attracted more than huts containing clean clothes. Willis (1947) reports that females of Aedes aegypti L. and

Anopheles quadrimaculatus Say are attracted by the odor of the human arm when the odor is presented at a temperature of 34°C and R.H. 70-85%. He also found CO_2 in concentration 1, 10 or 50% in the air not attractive to females of A. aegypti or Anopheles quadrimaculatus when tested in an olfactometer. Bates (1949) expects 'smell' to be the primary stimulus in guiding a mosquito in its search for food. He says: "Insects are known to have, in very many cases, an extraordinarily keen sense of smell. It is surprising in view of this to find an almost complete lack of experimental evidence to support the possible role of smell in governing the host-seeking of female mosquitoes." Rahm (1956) reports that CO_2 emitted by the skin does not determine attractiveness and remarks (1957) that human odor and sweat may play a part in the attraction of mosquitoes to the human hand. He reports that the attractiveness of an 'artificial arm' consisting of a warm bottle at 35°C covered with a moistened cloth, increased four times when an air stream carrying the odor of a human hand was added. It then equalled that of a normal hand. He concludes: "The odor of the human skin plays a significant role in the attraction, while sweat does not considerably increase it." Again in 1957 he reports that respiration does not seem to attract the mosquitoes but that the odors given out by the host do. Rahm (1958) speaks with absolute confidence when he says that the olfactory substances of man are shown to be alone responsible for greater activity of the female A. aegypti. Dethier (1957) in his review of the subject says: "Host finding and discrimination, trail following, orientation to odors by flying insects and courtship are shown to depend largely on chemical stimuli. Olfactory stimuli may be both orientating and releasing. Much of the confused understanding of various complex insect behaviour patterns can be attributed to a failure to appreciate the law of heterogeneous summation."

3. EXPERIMENTAL - EFFECT OF WASHING CHEMORECEPTORS WITH LIPOID SOLVENTS

3.1 Introduction

Amongst the advocates of chemical theories referred to under Section 2.2 many have suggested lipoid solubility as a basis of olfaction. Experiments were conducted to examine the effect of fat solvents on the antennal chemoreceptors of *Aedes aegypti* females in relation to the perception of stimuli from the host.

3.2 Methods and Materials

Ten female *Aedes aegypti* eleven days old were taken for each experiment. The mosquitoes, which were fed on sugar solution only, were taken in a sucking tube and chilled for 1.5 minutes at 15°F. They were then placed on a filter paper on top of a cold petri dish and their antennae were washed with lipid solvents applied with a fine camel hair brush. The mosquitoes were then transferred to a clean petri dish lined with filter paper in a one cubic foot cage and allowed to recover. Thirty minutes after the operation a hand was introduced into the cage and the number of landings of mosquitoes on it was recorded for a period of 15 minutes. Mosquitoes were shaken off gently on landing and were not allowed to feed on blood. The antennae of controls were rubbed with a clean dry brush.

3.3 Results

The observations are recorded in Table 2, which show that the number of landings decreased very significantly on washing the antennal chemoreceptors with the lipid solvents. The standard error of the mean was used to find statistical significance between the means. Whether the decrease in landings is due to the loss of lipids from the nerve endings, or due to the narcotic or anesthetic effect of the solvents is difficult to determine.

TABLE 2

The mean numbers of *Aedes aegypti* females landing on a hand in a 15 minute period after treatment of the antennae with lipoid solvents.

The figures are means of three replicates.

Control	Acetone	Ether
174 $\pm$ 8	52 $\pm$ 12	43 $\pm$ 13

4. EXPERIMENTAL - EFFECT OF REPELLENTS ON BEHAVIOUR

4.1 Blood Feeding in Relation to Repellents

4.11 Introduction

Christophers (1960, p. 486) remarks on the blood feeding behaviour of *Aedes aegypti* in the following words: "Another striking feature of feeding is that the insect, once it has begun to suck blood, appears to become oblivious of all danger and considerable physical force is required to make it give up its hold. Moreover, even when dislodged, it will often refuse to leave the spot and in spite of being pushed about will endeavour to bite again." This feature is referred to by Gordon and Lumsden (1939) when they note that they were only able to get *A. aegypti* to feed on the frog's foot by employing mosquitoes which had been allowed to start feeding on the human arm. When nearing repletion, however, the insect usually leaves readily if disturbed.

Kalmus and Hocking (1960) observed the feeding behaviour of *Aedes aegypti* in relation to repellents by painting the repellent with a fine camel hair brush on the backs of mosquitoes feeding on blood. A lead was taken from this study and some more observations were made on the effect of repellents on mosquitoes - both other species of *Aedes* in the field and *Aedes aegypti* in the laboratory, engaged in the act of feeding.

4.12 Observations on species of *Aedes* in the field

For studies on the species of *Aedes* in the field a thicket of poplar trees was selected. The same four repellents, dimethyl phthalate, ethyl hexanediol, indalone, and N-N-diethyl metatoluamide were used for the study. The mosquitoes reacted to all four repellents in the same way. The species of *Aedes* studied were *A. punctor* Kirby, *A. cataphylla* Dyar and *A. intrudens* Dyar.

(i) Feeding time: The time to take a complete blood meal, from the insertion of the proboscis to its retraction after complete engorgement ranged from two to four minutes. (Mean = 2 min. 31 sec. with standard deviation 41 sec.)

It was observed that the mosquitoes could be very easily disturbed in the early stages of their blood meal. If a clean artist's brush were brought near them soon after the insertion of the proboscis, they could be seen retracting it. If a repellent or olive oil were placed near the antennae or painted on the mesonotum, the mosquitoes invariably flew away. As reported by Kalmus and Hocking (1960, p. 7) "A contact between repellent chemicals as liquids and substantial areas of the proboscis, tarsi and tibiae, mesonotum or the wings leads to the interruption of biting, and in mosquitoes not engaged in biting to the retraction of the touched limb or limbs or to take off." But the behaviour of mosquitoes was found quite different in relation to repellents and other stimuli if they had been feeding for a minute or more, i.e. roughly in the middle of their meal; e.g.

(ii) The mesonotum was rubbed with a dry brush, painted with repellents or olive oil until the whole mesonotum was covered with liquid, but the mosquito never flew away, instead it completed its blood meal, continuing to feed for another 45 seconds to one minute.

(iii) The antennae were painted with repellents, were in fact soaked in repellent, but the mosquitoes continued to feed.

(iv) A drop of repellent was made to flow near the tarsi. No reaction was observeduntil it made contact with them. As soon as contact was made the tarsus was lifted. The same reaction was observed with olive oil. However, the mosquitoes continued to feed even when the tarsi of all the six legs were lifted. The mosquito then came to rest on its abdomen.

When the repellent was presented on a brush near the lifted tarsi, they sometimes rested the tarsi on the repellent soaked brush without showing any other abnormal behaviour and continued to feed.

(v) Similar behaviour was observed in mosquitoes feeding on the foot through socks. Mosquitoes coming to feed landed only on the clean portion of the sock and avoided the portion where repellent had been placed. However, the mosquitoes which were feeding through the sock for some time were not affected if a repellent was placed on the sock underneath them, and they continued to feed to completion although they lifted the abdomen.

(vi) Chloroform or ether was brought near the abdomen of a feeding mosquito. It always flew away, even when it had been feeding for a minute or more.

(vii) A hot spatula was brought near the mosquito (about 1 mm.). The spatula was heated for two minutes in a flame of a spirit lamp. Eighty per cent mosquitoes took off in 5 to 10 seconds. When the spatula heated for the same time was kept at the same distance from the mercury bulb of a Fahrenheit thermometer, the thermometer registered a rise of 4-6 degrees in 10 seconds.

(viii) Repellent was painted on the wing of a feeding mosquito. The mosquito always flew away but when the wing was rubbed with a dry brush or painted with olive oil it continued to feed.

(ix) Physical injury was inflicted on the mosquito to the extent that all the six legs were clipped off at the femoro-tibial joint, but it continued to feed and did not fly away.

The observations were made at a temperature of 65°F and R.H. of 57%.

4.13 Observations on *Aedes aegypti*

In the laboratory the same behaviour was studied in *Aedes aegypti*. A one cubic foot cage made of steel wire and covered with nylon net was fitted with a sleeve on each of two adjacent walls, i.e. at right angles to one another. Mosquitoes were allowed to feed on a hand inserted through one sleeve while the other hand was introduced through the other sleeve to apply the repellent, for examining the feeding behaviour in relation to it.

As observed in the other species of *Aedes*, *Aedes aegypti* could also be easily disturbed in the initial stages of blood feeding, but after one minute of feeding they could not be disturbed so easily:

(i) When their mesonotum was rubbed with a dry brush or painted with olive oil or any of the four repellents under study.

(ii) When their wings were painted with repellents. This was contrary to the behaviour observed in the field species which invariably flew away whenever repellents or olive oil were painted on the wings.

(iii) They continued to feed even when they were made to rest their tarsi on the repellent soaked brush.

(iv) Being small in size, it was not possible to paint their antennae with repellent while they were feeding, but when a drop of repellent was placed very close to the proboscis they continued to feed.

(v) Almost every mosquito continued to feed when the tarsi of its hind legs were clipped off, but some flew away when the tarsi of their other legs were clipped.

(vi) When a heated spatula was brought near them they always flew away even when the spatula was as far as 1-2 cm away, while in the field species of *Aedes* it had to be brought much nearer to the mosquitoes to

elicit this response. When the spatula heated for the same time was kept at the same distance from the mercury bulb of a Fahrenheit thermometer the thermometer registered a rise of 1.5 to 2 degrees.

4.14 Blood feeding after painting repellents on different chemoreceptor sites of *Aedes aegypti*

To study blood feeding in relation to repellents, experiments were conducted by applying the repellent on different sites of chemosensory fields of the female *A. Aegypti* and observing the behaviour and recording the number feeding on an untreated human arm. As the repellent was not applied on the skin, there was no interaction between the skin and the repellent or the chemical stimuli emanating from the skin and the repellent on the surface of the skin. The experiments provided some understanding on the site of action of different repellents as well as providing a quantitative basis for comparing the repellents with each other. The experiments also provide a quantitative basis for evaluating the function and efficiency with which the different chemosensory fields play their role in the act of feeding as well as some ground to accept the role of smell in attracting the mosquitoes to feed and the function of the repellent when applied on the skin in offsetting this role.

4.141 Methods and Materials

The experiments were conducted by taking 10-12 female mosquitoes, 7-8 days old, previously fed on raisins and sugar solution only, in a sucking tube and chilling them for 1.5 min at 15°F, in order to immobilize them. Their proboscides, either one or both antennae, or all the tarsi, were then painted with repellents on a fine hair brush in separate sets of experiments. This operation was performed over a cold petri dish covered with a filter paper and placed under a binocular microscope. A

radius was drawn in ink on the filter paper and mosquitoes were treated one by one, starting on one side of the radius until all of them were treated. They were then sucked back in the sucking tube and released in a paper lined petri-dish in a one cubic foot cage of steel wire with nylon net around it, to revive. The mosquitoes recovered from the chill in 2-3 minutes. The behaviour and the number that fed on blood on introducing the arm into the cage through a sleeve were noted, firstly ten minutes after the treatment and then at intervals of one hour from the treatment until the number fed in a given time corresponded with the number fed in controls. Two controls were run with each set of experiments, one a plain control when the receptor field that was intended to be treated was rubbed with a dry brush only, and another when it was painted with olive oil. The palps could not be treated separately without running some repellent on the proboscis and the antennae, because of their close proximity to these structures.

TABLE 3

Cumulative mean percentages of _A. aegypti_ females that fed after different chemoreceptor areas were painted with repellents. The figures are means of four replicates

Chemoreceptor area painted	Observation time	Control	Olive Oil	D.M.P.	D.E.T.	Rutger's 612	Indalone
Proboscis	10 min	82±3.1	73±2.5	33±3.6	12±3.9	22±5.5	10±3.5
	1 hr	--	--	72±4.4	51±8.7	52±3.7	47±3.2
	2 hr	--	--	75±4.7	72±2.9	76±5.7	63±2.6
	3 hr	--	--	--	--	--	71±1.6
Both Antennae	10 min	76±2.3	71±4.1	33±2.3	3±3.	0	3±3
	1 hr	--	76±2.5	81±4.2	6±3.2	5±2.8	8±4.8
	2 hr	--	--	83±2.3	11±4.1	11±4.1	19±5
	6 hr	--	--	--	32±3.1	33±4.5	30±3.1
One Antenna	10 min	85±2.9	80±4	61±3.3	5±2.7	25±5.9	19±3.3
	1 hr	--	--	80±4	38±10	49±4.3	56±2.4
	2 hr	--	--	--	75±2.3	69±3.2	72±4.5
Tarsi	10 min	80±4	73±2.5	27±2.7	14±5.2	27±2.9	30±3.8
	1 hr	83±2.5	78±2.5	50±8.4	61±2.3	71±3.9	74±3.5
	2 hr	--	--	68±1.5	76±2.5	76±2.2	79±1.5

4.142 Results

The figures given in Table 3 give the cumulative mean percentages of mosquitoes feeding on blood after different chemoreceptor sites were painted with repellents. The standard error of the mean was used to find statistical significance between the means.

The results show that Rutger's 612, diethyl toluamide and indalone reduce the number of mosquitoes feeding on blood more than dimethyl phthalate after the first ten minutes when the proboscis was painted and they remained effective for one hour, while the effect of dimethyl phthalate is lost in this period. Indalone remains significantly more effective as compared to Rutger's 612 and diethyl toluamide for up to two hours when it is painted on proboscis.

When painted on both the antennae, diethyl toluamide, Rutger's 612 and indalone reduce the number of mosquitoes feeding more than dimethyl phthalate. The effect of dimethyl phthalate was found to have been lost within one hour but the other three repellents remained highly effective even after six hours.

When painted on one antenna, the same significant differences were found between the repellents as was discovered when both the antennae were painted, i.e., diethyl toluamide, Rutger's 612, and indalone were significantly more effective than dimethyl phthalate.

The results obtained on painting all the tarsi with repellents were however different. Dimethyl phthalate was found to reduce feeding more effectively when painted on tarsi as compared to its effect when painted on both the antennae or proboscis.

Dethier (1956, p.1) remarks: "The evidence that many repellents work by way of specialized chemoreceptors is most convincing. Weismann

and Lotmar, 1949; Dethier and Yost, 1952; Peters, 1956; Dethier, 1956,b)". Peters (1956) reported that Calliphora erythrocephala could detect dimethyl benzamide with the tarsal receptors only while other materials like indalone and dimethyl carbate could be detected with the tarsal receptors, labellae, and antennae.

The significant difference in the number of mosquitoes feeding on blood after treatment of chemoreceptors on different head appendages and on the tarsi can be explained on the basis of the population of chemoreceptors getting such treatment. As most of the chemoreceptors are situated on the antennae, their treatment with repellents would inhibit the landing of mosquitoes on an arm more than the treatment of other head appendages. The ineffectiveness of the painting of one antenna only in keeping the mosquitoes from a blood meal for two hours can be explained by the same argument, i.e. a large population of chemoreceptors remained functioning effectively when only one antenna was painted. The painting of any one of these chemoreceptor sites with repellent must be affecting the mosquito in two ways, affecting the chemoreceptors of the chemosensory area painted in liquid form and also affecting the adjacent chemosensory sensilla in vapour form. The greater the area painted, the greater the number of sensilla affected, resulting in inhibition of feeding for a longer period.

4.15 Blood feeding after painting repellent on the mosquito-antennae and the arm of the host

By the same procedure as described earlier (p. 19) one antenna of each of about 10 A. aegypti females was painted with diethyl toluamide. An arm also treated with diethyl toluamide was then introduced into the cage and the behaviour of the mosquitoes was studied. A little more flight activity and some searching on the wing was observed in these mosquitoes as

compared to those in the control where no repellent was applied on the mosquitoes themselves but only on the hand. In controls, mosquitoes were seen sitting quiescently on the walls of the cage with little or no flight activity.

When both the antennae of mosquitoes were treated with diethyl toluamide and the same repellent was applied on the arm introduced into the cage, the mosquitoes could be seen searching on the wing. Many landed on the repellent coated surface of the arm, walked about and even probed but did not take a blood meal. The behaviour was observed for 10 minutes every hour for four hours but no mosquitoes bit.

4.2 Sugar Feeding

4.21 Introduction

The preferred food of an Aedes aegypti female is blood from a human host though it can exist for long periods on food other than blood. The male does not take blood at all but feeds entirely on nectar. Goeldi (1905) kept females alive for 31 to 102 days on honey alone. Macfie (1915) observed that the females feed on honey for the first couple of days but the males feed only on honey at anytime. Gordon (1922b) observed both males and females of Aedes aegypti sucking nectar from flowers. Many observers have noted that sugary fluids, raisins, bananas etc. are sucked by both sexes.

Many workers have devoted much time to studies of effect of repellent on blood feeding of mosquitoes but their effect on sugar feeding has not attracted much attention. Evans (1961) has studied the effects of several attractants and repellents on the ingestion of sugar solution by the blowfly Phormia regina Meigen. I conducted some experiments to study the effect of repellents on the feeding of Aedes aegypti on raisins.

Kalmus and Hocking (1960) conducted some tests on blood feeding in

relation to repellents with Aedes aegypti by keeping a 10 cm. length of 3 mm O.D. glass tubing which was clamped in a vertical position so that the lower end was about 1 cm above the middle of the 6 cm bare circle on the back of a gloved hand. A few drops of repellent were placed in the lower end of the tube. In this way a circle of skin about 1.5 cm diameter was kept free of bites. A lead was taken from this experiment in finding the effect of repellents on the feeding of Aedes aegypti on raisins.

4.22 Methods and Materials (i)

About 100 male and 100 female mosquitoes were taken in a cubic foot cage of steel wire covered with nylon net. The age of the mosquitoes was 2 - 4 days and they were not fed anything for six hours prior to experiments. Ten raisins were fixed with 1 cm clear space between each on a horizontal steel wire hanging 4 inches below the top of the cage. The wire was hung by bending its ends and hooking them on top of the side walls of the cage. A 2 cm wide strip of paper was fixed on top of the raisins, running parallel to them at a distance of 1.5 cm. Half of this paper strip (covering 5 raisins) was painted with repellent and the other half (covering the other 5 raisins) was kept as a control. Observations were made on the number of mosquitoes settling on either side at intervals of 5 minutes. After each observation the cage was shaken and another observation recorded after five minutes. In this way five replicates were taken for each repellent. Separate batches of mosquitoes were taken in separate cages for experiments with different repellents.

4.23 Results

The observations are recorded in Table 4. The vapour of repellents significantly reduced the number of mosquitoes feeding on raisins. The standard error of the mean was used as statistical test for significance.

TABLE 4

Mean numbers of *A. aegypti* settling on raisins separated by 1 cm, under the plain and repellent coated halves of a paper strip. The figures are means of five replicates.

Paper strip half	Olive oil	D.M.P.	D.E.T.	Rutger's 612	Indalone
Painted with chemical	18 ±1.9	5±0.5	3±0.7	3±0.1	2±0.8
Plain	19±4	18±1.9	12±1.3	13±1.4	10±2.2

4.24 Methods and materials (ii)

In a second set of experiments, 10 raisins were fixed on the wire lying as close to each other as possible without touching each other. Five alternate raisins were then painted with repellent leaving the other five as controls. The numbers of mosquitoes that settled on the treated and untreated raisins are recorded in Table 5. The figures are means of 5 replicates. Observations were recorded every five minutes as in the previous experiment. The total number of mosquitoes in the cage for each experiment was 200.

4.25 Results

In these experiments mosquitoes were seen coming close to the raisins to land but they usually flew away without landing. No significant difference was found between the number of mosquitoes settling on treated and untreated raisins in the control with olive oil. The results show that the repellent on the treated raisins kept the mosquitoes away from the untreated raisins as well. Kalmus and Hocking (1960, p. 23) obtained bites up to almost a mosquito halfwidth (about 2.3 mm) from a repellent painted circle on the back of the hand. In these experiments on feeding of mosquitoes on raisins the mean width of untreated raisin separating the two treated ones with repellent was 10 $\pm$ 0.3 mm.

The short distance of only a mosquito halfwidth from the repellent painted circle on the back of the hand where mosquitoes were seen feeding in experiments by Kalmus and Hocking (1960) was perhaps due to the factors of heat, moisture, CO_2 and probably skin odor which were missing as attractant factors in the raisins in these experiments.

TABLE 5

Mean numbers of A. aegypti settling on repellent treated and untreated raisins when placed close to each other The figures are means of five replicates

Chemicals	Untreated Raisins	Treated Raisins
Olive oil	16$\pm$2	14$\pm$4
D.M.P.	1	0
Rutger's 612	1	0
Indalone	0	0
D.E.T.	0	0

4.26 Methods and materials (iii)

In a third set of experiments the raisins were kept 1 cm clear apart from each other and alternate raisins were painted with repellent. Other factors were the same as in the previous experiments. The mean numbers of mosquitoes that landed on the treated and untreated raisins are given in Table 6.

4.27 Results

The numbers of mosquitoes feeding or settling on the untreated raisins were still very low and no significant difference was found in the number of mosquitoes feeding on untreated raisins in this experiment as compared to the number of mosquitoes feeding on untreated raisins in the previous experiment.

4.28 Methods and materials (iv)

In a fourth set of experiments only 5 raisins were taken and were placed 1 cm clear apart and the portion of wire between them was painted with repellent. Since the raisins were not painted with repellent in this experiment their number was reduced to five so that the number of mosquitoes landing on them could be compared with the number of mosquitoes landing on the untreated raisins in previous experiments.

4.29 Results

The results are given in Table 7. The comparison of results in Table 7 with those in Table 6 shows that significantly more mosquitoes settled on raisins in this experiment than in experiments where treated and untreated raisins were placed close to each other. This is perhaps due to the small quantity of repellent that could be applied on the small surface area of wire between the raisins as compared to the much greater area of the raisins in the previous experiments. However the repellents on the raisins themselves completely checked the settling of mosquitoes on them in experiments 2 - 4.

TABLE 6

Mean numbers of *A. aegypti* settling on repellent treated and untreated raisins separated by 1 cm. The figures are means of five replicates

Chemicals	Untreated Raisins	Treated Raisins
Olive oil	10 ± 0.7	11 ± 1.4
D.M.P.	2 ± 0.5	0
Rutger's 612	2 ± 0.8	0
D.E.T.	0	0
Indalone	2 ± 0.5	0

TABLE 7

Mean numbers of A. *aegypti* settling on raisins separated by 1 cm on a wire with a portion of wire between them treated with repellents. The figures are means of five replicates

Chemicals on wire	No. of mosquitoes settling
Nil	11$\pm$1.4
Olive oil	10$\pm$1
D.M.P.	2$\pm$1
Rutger's 612	3$\pm$0.5
D.E.T.	3$\pm$1
Indalone	5$\pm$0.5

4.3 Mating

4.31 Introduction

In Aedes aegypti "The stimulus which induces the male to copulate is the sound produced by the female during flight." "..odor plays no part in the sexual behaviour of aegypti..." (Roth, 1948, pp. 284, 282.) Roth also observes that in Aedes aegypti the male is the aggressor and is attracted by the female in flight and that the female is passive and does not show any mating behaviour similar to that of the male. "... never in our observations was a male seen to initiate copulation with a resting female" (Roth, 1948, p. 276). Banks (1908, p. 246) on the contrary stated that specimens of aegypti confined in small jars "... have been seen to copulate while the female hangs from the gauze covering the vessel, the male always approaching her from the ventral surface." Christophers (1960, p. 502) observed that copulation takes place quite commonly with the female at rest. During the course of this work it was observed that a female Aedes aegypti is not entirely passive and that copulation does take place when a female is at rest. It was observed that when a flying male came close to a sitting female, the female would take flight and the male would grasp it for copulation. Many times females were seen taking flight spontaneously and males were seen getting hold of them in mid air. The males were also observed coming to land sideways with a female, then trying to take a ventral position and many a time they succeeded. At other times because of his efforts to gain a ventral position to the resting female the male roused the female to fly and copulation took place on the wing or the two could be seen falling on the floor copulating. But mostly copulation took place with a female in flight.

Roth (1948) also observed that the male would copulate repeatedly with the same or different females. After repeated matings, females

become more and more reluctant to fly and would resist the attempts of the males to copulate. Richard (1927) suggested that repeated copulations exhaust the individuals. In view of this observation it was necessary to separate the sexes before they started mating and to keep the observation time reasonably short. Shannon and Putnam (1934) in their laboratory study of *aegypti* observed that the average pupal period of females was 14 hours longer than that of males. Roth (1948, p. 308) observed that by the time the female begins to fly and becomes 'attractive' the male's antennae have reached a state where the sound stimulus can be perceived and his genitalia have rotated sufficiently so that copulation can be successful (usually about 15 to 24 hours after emergence). Thus to prevent the element of fatigue in the females, the males were separated from the females 14 hours after emergence.

4.32 Methods and materials

Ten females 2 - 4 days old and 10 males 5 - 6 days old were used for each experiment. The females were chilled in a sucking tube for 1.5 minutes at 15°F and then all their tarsi were painted with repellent with a fine brush while on a cold petri dish under a binocular microscope. Since *Aedes* mate venter to venter and during coitus the female *aegypti* does not clasp the male to her, her legs remaining out-stretched, merely serving as structures to which the male clings, (Roth, 1948, pp. 270,301) it was decided to paint the tarsi of the female mosquito with repellent. After the tarsi were painted the females were released in a one cubic foot cage and allowed to recover from chill. Ten minutes after the treatment (the females had recovered in 3 - 4 minutes) 10 males were released in the cage. After application of the repellent on the tarsi of the female *A. aegypti* few flew spontaneously. Most females sat quietly on the walls of the cage. Males hardly ever succeeded in persuading the female at rest

to copulate. It was also observed, though no quantitative basis could be laid down for this observation that the efforts of the male to copulate with the resting female as well as with the female in flight were less persistent and quite often they were seen releasing the female soon after coming in contact. The cage was therefore shaken every minute to make the females fly and the number of matings in a period of 30 minutes was recorded. Each experiment was performed with a new batch of mosquitoes.

4.33 Results

The results are recorded in Table 8. The standard error of the mean was used as a test of significance. The highly significant reduction in the number of matings in _A. aegypti_ in association with repellents can be explained as a result of two factors: 1) a decrease in the flying activity of the females and 2) less persistant efforts and premature release of the female. The males were observed releasing the repellent treated females soon after coming in contact with them without copulating.

Though the cage was shaken every minute in experiments with repellents as well as in the control it was observed that the females in the controls continued to fly for a much longer time after shaking than in experiments with repellents. With repellents, most of the time the females could be seen coming to rest on the wall immediately after shaking the cage, and many a time on shaking they would fly only from one wall of the cage to another. The lack of spontaneous flight activity on the part of the female was another factor which combined with the decrease in flying activity on application of the repellent resulted in the reduction of the number of matings significantly.

TABLE 8

Mean numbers of matings in a 30 minute period in a population of 10 male and 10 female *Aedes aegypti* with repellents applied to the tarsi of the females. The figures are means of four replicates

Control	Olive oil	D.M.P.	Indalone	Rutger's 612	D.E.T.
65±2	65±1	33±2	30±3	33±3	33±3

4.4 Oviposition

4.41 Introduction

Wallis (1954) in his studies on the oviposition activity of mosquitoes, including _A. aegypti_, found that the female could detect an objectionable amount of salt even when the movements of the abdomen were restricted. Likewise surgical removal of the palpi, proboscis, and antennae from the head did not result in loss of sensitivity. Surgical removal or wax coating of various combinations of legs and leg articles resulted in the demonstration that sensitivity was localized in the tarsal articles of all the species of mosquitoes studied by him. His investigations also showed that the sensitivity was present in all the tarsal articles of _Aedes aegypti_. The thin walled chemoreceptors of the tarsi enabled the mosquitoes to detect differences in saline concentrations as slight as 0.02 M.

Brown (1960) studied the role of olfaction in the stimulation of oviposition in the Blowfly _Phormia regina_ Meigen. He found that the odor of a liquid medium containing powdered milk and yeast stimulated the blowfly to oviposit. He also provided evidence for olfactory perception by the ovipositor of the blowfly.

The study of oviposition activity of mosquitoes by Wallis (1954) mainly concerned the perception of salt concentration in water by the chemoreceptors on the tarsi of the mosquito. In this study oviposition in _Aedes aegypti_ was observed by associating oviposition sites with repellent vapours as well as by applying repellents on the tarsal chemoreceptors.

4.42 Methods and materials (i)

Five, 7 - 8 day old blood fed females in a one cubic foot cage were taken for each experiment. The cage was provided with a rectangular platform, 7" x 4" made of a steel wire frame (diameter of wire 2.5 mm). The

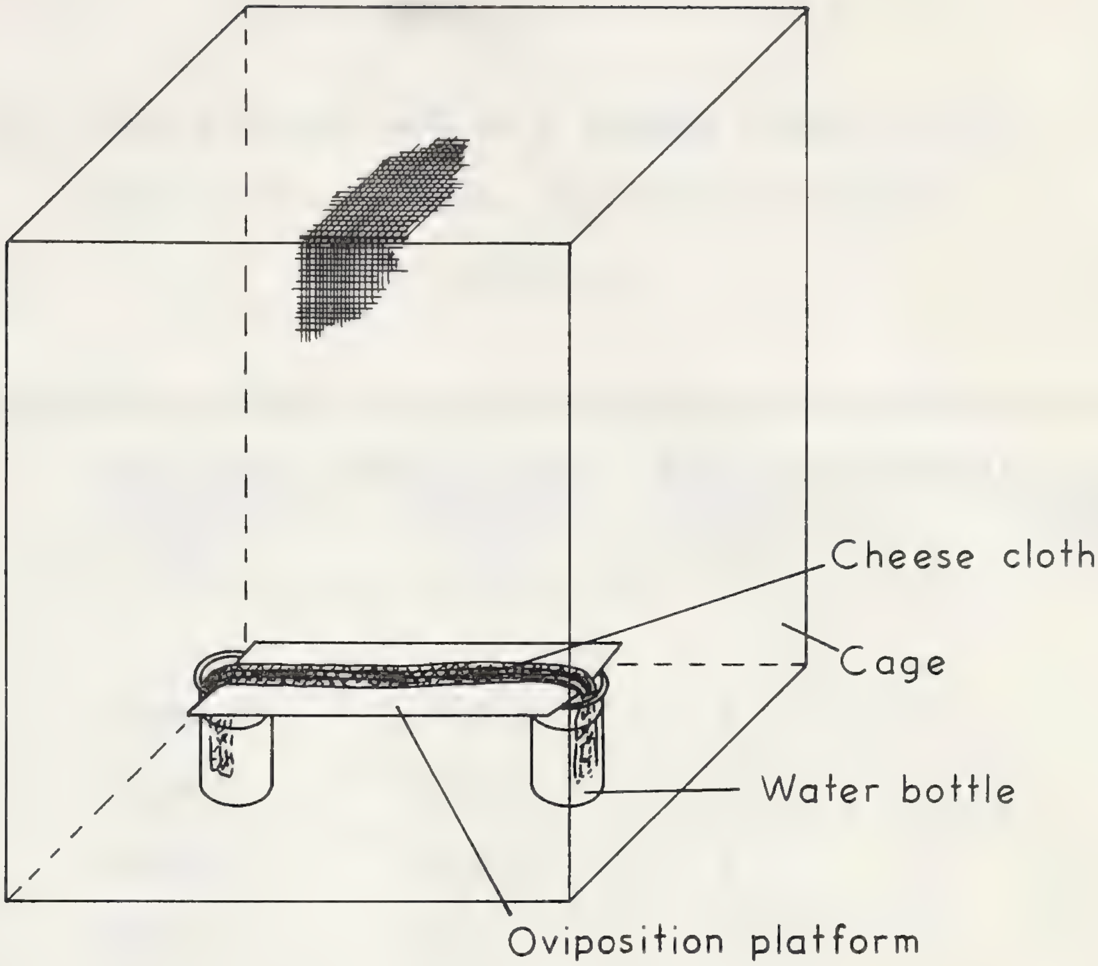
platform was covered with nylon net on one side and with two paper towel strips pasted on the other except in the centre where a gap of 1 cm was left in between the strips, Figure 1.

The platform was placed in the cage, nylon-net-side upwards on two glass bottles filled with water. On the nylon net was spread a piece of cheese cloth, the two ends of which remained dipped in water in glass bottles on the sides on which the platform rested. The cheese cloth was kept wet by capillary action by the water in the two bottles. One of the two paper strips was painted with repellent while the other was left untreated. Thus an oviposition platform for the mosquitoes was provided, one half of which had repellent vapour coming from underneath through the nylon screen, while the other half served as control. The nylon net underneath the cheese cloth served as a support for it and did not allow it to come in contact with the repellent on the paper strip below but allowed the repellent vapours to pass through. Most of the eggs were found to be laid on the cheese cloth but some were laid on the paper strip. They were counted separately 72 hours after the blood meal, and the results are recorded in Table 9. Four experiments were run with each repellent.

4.43 Results

The behaviour of Aedes aegypti during egg laying is described in detail by Wallis (1954). During the experiments it was observed that a female mosquito could sample the oviposition sites while on the wing and would land on the control half rather than on the repellent treated half of the oviposition platform. At other times when she landed on the repellent half it walked for a few seconds and then flew away and landed on the control side. This behaviour demonstrates the function of olfactory receptors in the selection of an oviposition site when repellent

a.



b.

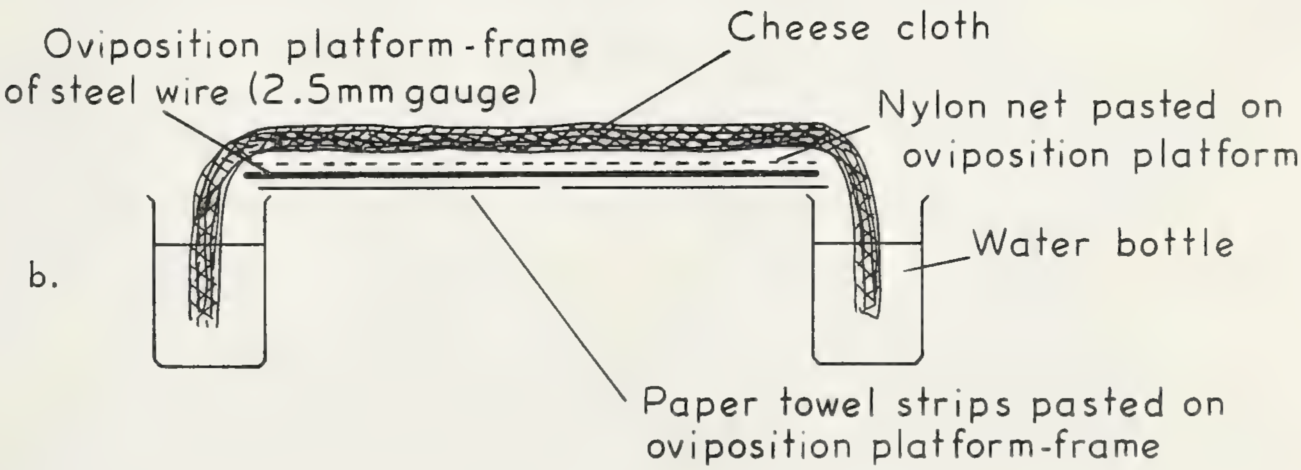


Figure 1. Diagrams showing: (a) arrangement of the oviposition platform in the cage, and (b) a vertical section of the oviposition platform.

TABLE 9

Mean numbers of eggs laid by A. aegypti females in the presence of repellents. The figures are means of four replicates

Chemicals	Eggs laid on control side		Eggs laid on repellent side	
	On cheese cloth	On paper towel	On cheese cloth	On paper towel
Rutger's 612	128±18.6	52±4.2	0	0
D.E.T.	160±20.5	47±2.2	2±1.7	0
Indalone	156±18.4	19±1.5	0	0
D.M.P.	208±13	22±1.7	5±1.1	0
Olive oil	87±6	12±1.1	68±7.2	0

vapours are associated with it. The complete absence of egg laying on the repellent coated as well as olive oil coated paper towels on the lower side of the platform seems to be the result of tarsal chemoreceptors which select the suitability of the egg laying medium on contact. The significantly small numbers of eggs laid on cheese cloth on the repellent side as compared to the number of eggs laid on the control side show that Aedes aegypti rejects oviposition sites when these are associated with repellent vapour.

4.44 Methods and materials (ii)

Experiments were also conducted by painting the tarsi with repellent by the same technique as described in previous experiments and recording the number of eggs laid in 24 hours. Christophers (1960, p. 507) records that egg laying in Aedes aegypti usually begins on the afternoon of the third day from blood feeding, counting the day of feed as zero. Female mosquitoes 6 - 7 days old were fed on blood and left in a cage with raisins for three days, taking the day of feed as zero. On the fourth day their tarsi were painted with repellent and the mosquitoes were placed singly in separate vials with water soaked cotton wool in the bottom and a nylon net cap on the top on which was placed a raisin. Eggs laid in a 24 hour period were then counted. Four replicates were run for each experiment. The mean numbers of eggs laid are recorded in Table 10.

4.45 Results

The difference in the number of eggs laid in control and those laid in relation to repellents is not significant, using standard error of the mean as a test of significance.

4.46 Methods and materials (iii)

Experiments were also conducted to determine whether antennectomized mosquitoes would discriminate between the control and the repellent

TABLE 10

Mean numbers of eggs laid by single *A. aegypti* females after painting the tarsi with repellents. The figures are means of four replicates

Control	Olive oil	D.M.P.	Rutger's 612	D.E.T.	Indalone
39±8.8	42±4.8	29±5.2	34±8.6	34±5	33±4.4

sides of the oviposition site and for this purpose 20 female mosquitoes which had been fed on blood previously were operated upon for each experiment on a cold petri-dish under a binocular microscope after first chilling them for 1.5 minutes at 14°F. 10 to 12 flagellar segments of the antennae were excised and the mosquitoes then released in the cage with the oviposition platform as described in previous experiments.

4.47 Results

Though sometimes mosquitoes could be seen sitting on the control side of the egg laying platform, no eggs were laid in any of the experiments over a week's time except in the experiment with diethyl toluamide where there were 4 eggs on the control side. A high mortality (70 - 75%) was also observed in mosquitoes during this period. The almost complete absence of oviposition by antennectomized mosquitoes may be due to lack of orientation of mosquitoes to the water soaked cheese cloth on account of the great reduction in the number of hygroreceptors as a result of excision and consequently a great increase in the threshold of moisture perception. The high mortality rate can also be assigned to the same factor i.e., lact of orientation to the water soaded cheese cloth and hence dehydration. Mosquitoes were seldom seen sitting on the wet cheese cloth. Most of the time they were found sitting on the walls of the cage with very little flight activity. The very low activity in antennectomized mosquitoes confirms the findings of Bar-Zeev (1960) who found only 4 per cent of mosquitoes could be activated when antennectomized as compared to 60.1 per cent when intact.

4.48 Methods and materials (iv)

Experiments were also conducted to test oviposition after treating the terminalia of the females with repellent. The female <u>aegypti</u> mosquitoes

were fed on blood when 7 - 8 days old, and their terminalia painted with repellent 72 hours after the flood feed by the same technique as described in the previous experiments and then released in the cage.

4.49 Results

All the mosquitoes became too crippled to move about or fly shortly after the painting of the tip of abdomen and died in a few hours.

4.5 Behaviour in Relation to Wind Direction and Speeds in the Presence of Repellents

Kalmus and Hocking (1960 p. 21) conducted a series of experiments in which target areas were drawn out on the backs of subjects who wore shirts with the backs cut out. They recorded the distribution of bites for a small repellent treated area. To demonstrate the effect of wind direction on the distribution of bites in relation to repellent, experiments were conducted in the laboratory on Aedes aegypti using the same technique.

4.51 Methods and materials (i)

A circle of 3.5 cm radius was drawn in hard clear nail varnish on the bare chest of a subject. Concentric to this another circle of 6.5 cm radius was drawn. The outer circle was divided into two equal halves by drawing a diameter at right angles to the direction of the wind. A hair drier was used to produce the air current and variable transformer was included in the circuit to permit adjustment of the speed of the wind. The wind speed was kept at 43 cm/sec. The source of wind i.e. the nozzle of the blower was kept 23 cm away from the central circle which was coated with repellent. The blower was kept in such a position as to give a uniform flow of air over the marked area. The repellent used was diethyl toluamide. One hundred 7 - 8 day old female Aedes aegypti mosquitoes were taken in a one cubic foot cage of steel wire with nylon net around it for each experiment.

The mosquitoes were fed on sugar solution only before the experiment. The cage was placed on the marked area and the portion of skin outside the marked area was covered with a polyethylene sheet. Mosquitoes soon started biting through the nylon net on the floor of the cage. An observer kept a record of the mosquitoes that settled and flew away, or settled and bit, in the upwind and downwind halves of the circle. The counts were made for 5 minutes in each experiment. Controls were run with the same wind speed without repellent. The number of mosquitoes that settled or bit in the upwind and downwind halves of the circle are given in Table 11.

4.52 Results

The results show that in the control where repellent was not painted in the central circle, significantly more mosquitoes settled or bit on the downwind side of the circle than on the upwind side. This is in conformity with the observations made by Kalmus and Hocking (1960, p. 4) with field mosquitoes. However, when repellent was painted in the central circle it was observed that the number of mosquitoes settling or biting on the downwind half of the outer circle was significantly lower then the number settling or biting on the upwind half. This was due to the presence of repellent vapour carried by the wind on the downwind half of the outer circle.

4.53 Methods and materials (ii)

In another set of experiments the effect of different wind speeds was determined on the settling and biting of mosquitoes in relation to repellent. Experiments were conducted in a similar fashion as described under the experiments with different wind directions, except that the portion of the body used was the thigh instead of the chest, which gave the advantage of the subject himself making notes of the number of mosquitoes landing or biting. A control was run with each wind speed and

TABLE 11

Mean numbers of *A. aegypti* females settling or biting in relation to wind direction and D.E.T. on the marked area of skin

Wind speed	Control* Upwind	Control* Downwind	D.E.T.** Upwind	D.E.T.** Downwind
43 cm/sec	15±2.5	27±1.6	35±2.9	8±1.7

* N = 2

**N = 3

all the controls with different wind speeds were run first in order to avoid contamination of skin area with repellent vapours. After the controls were run, different batches of mosquitoes were then used in experiments with the same wind speeds in relation to repellent painted in the central circle. The repellent used was diethyl toluamide. The portion of skin outside the outer circle was covered with polyethylene sheet and the count of mosquitoes settling or biting in the upwind or downwind half of the circle was recorded for five minutes in each experiment.

4.54 Results

The results are shown in Table 12. In previous experiments with different wind directions the number of mosquitoes settling or biting in the upwind half of the circle in experiments with repellents was significantly higher than the number of mosquitoes in the downwind half of the circle. The results given in Table 12 show that the mosquitoes continue to show the strong tendency of settling more on the upwind side in relation to repellent with different wind speeds.

The maximum wind speed at which mosquitoes were able to settle on a bluff body was reported to be 95 cm/sec and that of settling on the streamlined body to be 55 cm/sec. Kalmus and Hocking (1960, p. 15). In this case the maximum speed of wind at which the mosquitoes settled on the skin was 265 cm/sec which is very high as compared to the wind speed with the bluff or streamlined bodies. This is probably due to the attractant factors of the skin acting on the mosquitoes.

4.6 Orientation to Gravity and Centrifugal Force in Relation to Repellents

4.61 Methods and materials (i)

To study the orientation of *Aedes aegypti* to gravity in relation to repellents, experiments were conducted in a plastic petri dish of 9 cm diameter.

TABLE 12

The number of *A. aegypti* females settling or biting in the upwind or the downwind half of a circle marked on skin in relation to different wind speeds and D.E.T.

Wind speed	Control		D.E.T.	
	Upwind	Downwind	Upwind	Downwind
0 cm/sec	27	29	23	19
43 cm/sec	13	26	41	12
134 cm/sec	16	31	9	4
190 cm/sec	5	14	3	1
227 sm/sec	4	8	5	0
265 cm/sec	4	6	2	0
314 cm/sec	0	0	0	0

The lid of the petri dish was perforated with 2 mm diameter holes, about 9 holes per one sq cm, to allow the repellent vapours inside the dish to escape. The floor of the petri dish was lined with a filter paper which was divided into four quadrants designated top, left, bottom, and right.

Twenty female mosquitoes, 7 - 8 days old were taken, chilled for 1.5 minutes at $14^{o}F$ and then released in the petri dish. On recovery of mosquitoes from chill the petri dish was kept vertical and given five complete turns on its horizontal axis, and thereafter the position and the number of mosquitoes was noted in each quadrant after a minute. The experiment was replicated five times without repellent as a control. A band of repellent 1 cm wide was then painted on the outer margin of the top-quadrant. Mosquitoes were chilled and placed in the petri dish and allowed to recover. After the mosquitoes had completed recovered, the dish was given five complete rotations as in the control, keeping it vertical and rotating it about its horizontal axis. The experiment was repeated five times with each repellent.

4.62 Results

In the control the mosquitoes could be seen walking upwards and most of them collected in the top-quadrant. Significantly less mosquitoes remained in other quadrants. Almost all the mosquitoes were seen facing upwards and the root mean square deviation of their body axes from the vertical axis of the petri dish was found to be zero.

With repellent significantly less mosquitoes entered the top-quadrant. Most of them remained in quadrants-left, right and bottom. They were also seen walking at an angle to the repellent or turning away from it. Their angle of turning (i.e., the angles which the vertical axes of the bodies formed with the vertical axis of the petri dish) was noted by marking their position in each quadrant on a separate sheet of paper and then

measured the angle and direction of deviation from the vertical.

Table 13 shows the distribution of mosquitoes in the various quadrants of the petri dish in relation to repellents, while Table 14 shows the root mean square of the angle of deviation (in degrees) of the body axes of mosquitoes from the vertical axis of the petri dish in relation to repellents.

Results with olive oil were not found to be significantly different from those of the plain control.

The effect of the presence of repellent on the head upwards orientation of the mosquitoes in relation to gravity was found to be highly significant.

4.63 Methods and materials (ii)

The effect on geotaxis was also observed by painting repellent on the mesonotum and the antennae. 7 - 9 days old female mosquitoes were chilled for 1.5 minutes at 14^{o}F. and their mesonota or antennae were painted with repellent. They were then placed in a petri dish similar to the one used in previous experiments, having minute holes in the lid and lined with filter paper. After complete recovery of the mosquitoes from chill the dish was kept vertical, and rotated on its horizontal axis slowly, one rotation in 20 seconds, and the positions of the mosquitoes were noted. A similar study was first carried out by Kalmus and Hocking (1960, p.8) with normal mosquitoes in a 9 cm petri dish on a vertical turn table, rotating once in 20 seconds about a horizontal axis. Female _A. aegypti_ showed a counter rotation to maintain a head upward position.

4.64 Results

The mosquitoes with their mesonota painted oriented facing upwards but when antennae were painted with repellent, and placed in a 9 cm petri dish lined with filter paper; on placing the dish in a vertical position

TABLE 13

Mean numbers of *A. aegypti* females found in different quadrants in relation to repellents. The figures are means of five replicates

Chemical	Quadrants			
	Top	Left	Bottom	Right
Control	15±1	2±0.5	1±0.4	2±1
Olive oil	13±1.6	3±0.8	2±0.5	2±0.7
D.M.P.	3±0.4	4±1	4±1	9±1.3
D.E.T.	2±0.1	6±0.8	6±1.4	6±0.8
Indalone	4±1	5±1.2	6±1.4	5±1.3
Rutger's 612	2±0.5	5±0.4	8±1.3	5±1

TABLE 14

Root mean square of angles of deviation (in degrees) of the body axes of *A. aegypti* from the vertical axis of the rotating petri dish in relation to repellents.

The figures are means of five replicates

	Control	Repellents			
		D.M.P.	D.E.T.	Rutger's 612	Indalone
Angle in degrees	0	43±8.4	47±13.3	50±5.4	47±13.3

the mosquitoes could be seen sitting on the vertical surface head upwards cleaning their antennae with the tarsi of the forelegs. During this act the weight of the body was supported by the middle and the hind pairs of legs. When the dish was rotated slowly while they were cleaning their antennae, they did not seem to be bothered much until their face turned downwards. Then they were found to lose their balance and were seen to place their forelegs on the vertical surface. Some of them turned around, faced upwards and started cleaning the antennae again, but the typical counter rotation as observed by Kalmus and Hocking (1960) in their experiments was absent.

4.65 Methods and materials (iii)

The orientation of Aedes aegypti on centrifugation was studied by Kalmus and Hocking (1960, p. 8). According to them when mosquitoes were centrifuged in a 9 cm petri dish at 390 r.p.m. and observed under stroboscopic illumination, they were found facing towards the centre of the dish, and sometimes walking towards it.

In this study of the same behaviour in relation to repellents a plastic petri dish of 9 cm diameter was lined with filter paper on which one radius was drawn in ink. Its lid was extensively perforated by small holes. Mosquitoes, both males and females (50 to 60 adults) were released in this dish and centrifuged at 390 r.p.m. on a turn table and observed under stroboscopic illumination. Mosquitoes were seen as reported by Kalmus and Hocking (1960) facing towards the centre and walking towards it. It was also observed that most of them collect near the centre roughly 1 to 1.5 cm from it, and comparatively fewer mosquitoes remained at the periphery. The centrifugal force at 1 cm from centre was 1.7 g, and at 1.5 cm 2.5 g. As the dish continued to rotate more mosquitoes could be seen moving towards

the centre. For experiments with repellents the mosquitoes were taken in batches of 15, in a sucking tube, chilled for 1.5 minutes at $14^{o}F$ and then their mesonota painted with repellent on a cold petri dish under a binocular microscope. Dimethyl phthalate and diethyl toluamide were tested. After treatment the mosquitoes were released in a cage and allowed to recover. They were then introduced in the petri dish (50 - 60 of them) and made to rotate.

4.66 Results

Under stroboscopic illumination it was observed that the mosquitoes did not collect in greater numbers near the centre of the dish and the movement towards the centre was less noticeable. The whole dish gave an appearance of a scattered distribution of mosquitoes as compared to a circular distribution near the centre in the control. Quite a few (10 - 15%) faced directions other than the centre.

In another experiment the mosquitoes themselves were not treated but a circle of 4 cm diameter (centrifugal force 3.5 g) was painted with repellent in the centre of the dish. Mosquitoes (50 - 60) were introduced in the dish and made to rotate. It was observed, that, with an exception of one or two, the mosquitoes remained outside the circle facing towards it, and sometimes seen turning away from it, or walking around it. In yet another experiment when the diameter of the circle painted with repellent was increased to 6 cm (centrifugal force about 5 g) in 9 cm petri dish the same behaviour was observed. Most of the mosquitoes remained outside the circle, although the non-treated peripheral belt around the repellent coated circle was only 1.5 cm wide.

4.7 <u>Visual Responses in Relation to Repellents</u>

4.71 Introduction

The optomotor and visual responses of mosquitoes have been studied

by many workers. Kalmus (1958) reported that A. aegypti shows responses to the rotation of the plane of polarisation of light. In a later study Kalmus and Hocking (1960, p. 19) observed swarming flight in A. aegypti close underneath a weak light source placed on top of a darkened cage, but the same was not observed when a much stronger light was made to pass through a red filter. Mosquitoes were also observed by these workers to aggregate near the margins of black objects when these were placed on top of a weakly illuminated cage.

The visual response of mosquitoes was also studied by Kennedy (1939) and Rao (1947). Kennedy reported that suspended mosquitoes orientated accurately towards a vertical black stripe on a white background. Presented with two stripes the mosquitoes faced one or the other stripe and not between the two. Rao (1947) reported similar findings with Anopheles maculipennis atroparvus van Thiel, Culex (Culex) molestus Forskal rendered flightless by the removal of wings or by sticking them together.

4.72 Methods and materials

To test the effect of repellents on the visual response of Aedes aegypti to black stripes, 20 female mosquitoes were taken in a glass bottle 12 cm tall and with a diameter of 3 cm. The inside of the bottle was lined with white nylon net to give the mosquitoes a good foothold. This bottle was placed inside a glass cylinder 14 cm high and with a diameter of 6 cm. The bottle and the cylinder were placed on a thick glass plate which was resting on a tripod stand. Under the glass was placed an electric lamp (40 W) which was covered all around with a cylinder of black paper so that light could go only upwards and light the bottle and the cylinder outside it uniformly from inside. In order that the inside of the cylinder be evenly illuminated, a filter paper was placed on the glass plate on which

the outer cylinder and the inner bottle rested. The outer cylinder was divided into four quadrants and the alternate two quadrants were covered with black paper strips each covering 90°. The remaining two quadrants were left uncovered, Figure 2.

4.73 Results

As the outer cylinder was placed around the inner bottle containing mosquitoes and kept there for a short time, the mosquitoes inside moved and came to rest on the wall of the bottle facing the black stripes. The outer cylinder was then rotated 90° so that all the mosquitoes now faced uncovered portions of the cylinder. The mosquitoes moved again in the direction of the black stripes and again came to rest opposite to them. This behaviour could be observed again and again. However, when the antennae were painted with repellent they showed complete indifference to the black stripes and did not move towards them as in the control.

a.

b.

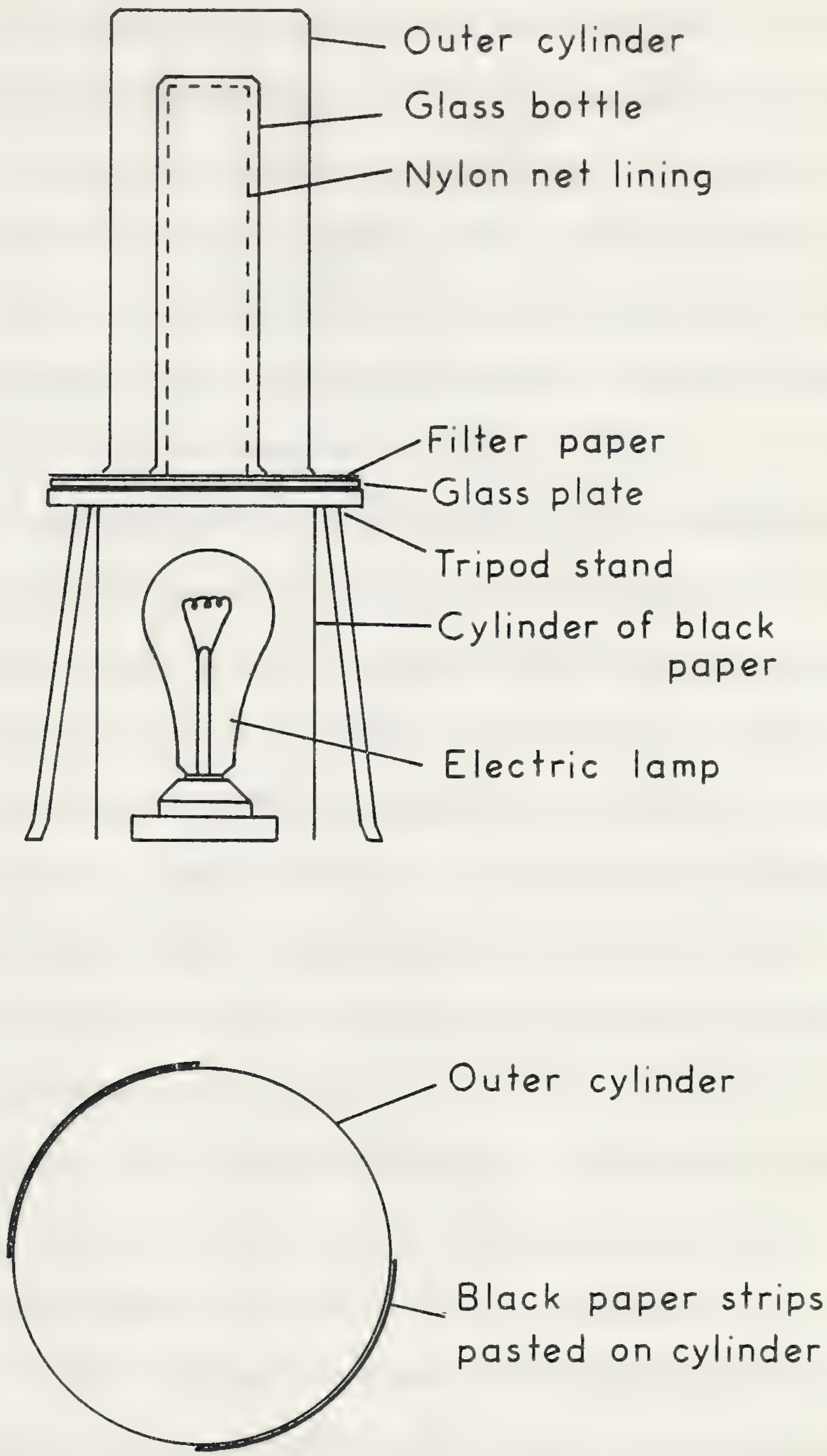


Figure 2. Diagrams showing: (a) a vertical section of the apparatus used for testing the visual response of *A.aegypti* females to black stripes in relation to repellents, and (b) a cross section of the outer cylinder.

5. DISCUSSION

The action of repellents on mosquitoes has been found to have no specificity for blood feeding behaviour. Repellents affect all the activities of mosquitoes. It has been shown that repellents in the vapour phase have the following effects on Aedes aegypti. They inhibit feeding on both blood and sugars, reduce the mating rate, and cause rejection of oviposition sites. The repellents also affected orientation to gravity and centrifugal force and the visual response to black stripes.

Mosquitoes became quiescent and less active when repellents were applied on them. This slowing down of motor activity suggests that the external stimuli normally acting on the mosquito are perhaps blocked or interfe with by the repellent. As there is no delay in the effect of repellents on the behaviour of mosquitoes, that is, protection is obtained immediately these materials are applied, their action on the insect may be assumed to occur at the surface of the body. Repellents have not been shown to penetrate rapidly into the body where they could act on the nerve synapses or the central nervous system, nor have they been shown to affect the muscular system directly. It thus seems unlikely that they act by blocking the nerve impulses or the motor response. The most probable action seems, therefore, to be the blocking of reception of stimuli at the receptor site.

Somewhat different behaviour in relation to repellents of another kind has been described by Kennedy (1947). He studied the effects of contact with DDT on the activity and distribtuion of mosquitoes. He argued from his experiments that a variety of reactions may give rise to repulsion. Reactions may occur at a distance or only after contact with a repellent surface. The contact stimuli may be mechanical or chemical. The reactions may take the form of an increase of merely random activity or they may be directed away from the surface. They may be quick

or slow to appear and weak or strong in expression. In contrast to my findings of reduced activity in his work an increase in activity is found.

The factors that attract mosquitoes to the host have been discussed in Section 2.3. The mode of action of insect repellents can be best understood when studied in relation to these factors.

The effects of repellents on the evolution of carbon dioxide and moisture from a human arm, and the correlation of this evolution with the natural attractiveness of human beings and protection time of repellents were studied by Gouck and Bowman (1959) at Orlando, Florida. In their experiments, repellents applied to the arms of three subjects reduced the CO_2 emitted by 9 to 14 per cent but they concluded: "Although these reductions are considerably greater than the differences between untreated arms (4%) they are not great enough to indicate that the mode of action of these repellents is based upon the retardation of carbon dioxide evolution." The repellents used were, dimethyl phthalate, diethyl toluamide and ethyl hexanediol. With regard to the moisture collected from untreated and repellent treated arms they concluded: "The quantities from the arms of all subjects varied from day to day but in most individual tests the two arms agreed within about 5 per cent indicating that no real difference in the amount of moisture evolved was caused by application of repellents." They believed that the protection time is governed by the rate of loss of repellent from the skin by absorption and evaporation. Peters and Kemper (1958) have shown that there are no considerable temperature differences between repellent treated and untreated parts of the skin.

In the light of these findings it can be said that repellents affect the reception of these stimuli rather than the stimuli themselves. This supports the hypothesis advanced that repellents affect many kinds of

behaviour of mosquitoes by interfering in the reception of many different kinds of stimuli.

Search for chemical factors other than carbon dioxide attracting mosquitoes to the host has claimed the attention of many workers. The findings of Shaerffenberg and Kupka (1951) and Burgess and Brown (1957) have indicated that attractive factors other than carbon dioxide are present in the vapour from mammalian blood and body exudations. A distillate obtained from mammalian blood by Shaerffenberg and Kupka (1959) proved highly attractive to Culex pipiens L. Rudolfs (1922) found benzoic acid, dilute ammonia, phenylalanine, alanine, aspartic acid, cystine, and hemoglobin to be attractive to Aedes sollicitans Walker and Aedes cantator Coquillett, but Reuter (1936) found the last six materials unattractive to Anopheles maculipennis atroparvus. Brown and Carmichael (1961) reported that L-lysine and L-alanine were attractive to Aedes aegypti. The effect of repellents in association with these chemicals found to be attractive remains to be studied.

Travis and Smith (1951) evaluated dimethyl phthalate, indalone, and ethyl hexanediol against Aedes aegypti besides other mosquitoes, and found the average repellent time (i.e., time in minutes from application of the repellent to the first bite) as follows: ethyl hexanediol - 331 minutes, dimethyl phthalate - 247 minutes, and indalone - 111 minutes. Although the results of my experiments are not strictly comparable with those of Travis and Smith (1951) for I worked with a different culture of mosquitoes and at a different time and place, the mosquitoes fed on blood much sooner after treatment when repellents were applied on the mosquito receptor sites. For example about 33 per cent of mosquitoes fed on blood within 10 minutes after application of dimethyl phthalate on both antennae. When diethyl toluamide, indalone, and ethyl hexanediol were separately applied on both

antennae, some of the first bites were recorded after 10 minutes. The reason for this behaviour is perhaps the more rapid adaptation of the receptors to the repellents because of the greater concentration gradient resulting from their application on the receptors themselves. In this way the threshold for reception of repellents increased greatly but that for the other stimuli remained the same. The sequence of stimuli and responses leading to blood feeding therefore remained unaffected. But this is, of course, incompatible with the hypothesis that repellents block all receptors.

The presence of separate chemoreceptor neurons mediating acceptance and rejection is assumed from the study of labellar chemoreceptor cells of *Phormia regina*. These cells have been the subject of co-ordinated behavioural, histological, and physiological study. A chemosensory hair of the labellum of this blowfly was described by Dethier (1955) as a hollow extension of the body cuticle possessing two distinct lumina. The chemosensory hair has been shown to be associated with three bipolar neurons, two of which send distal fibres to the terminal papilla by way of the thick-walled lumen of the hair. Dethier (1955) concluded that one of these neurons mediates acceptance while the other mediates rejection. On electrophysiological studies one of the two neurons was later designated the L fibre (for large spikes which responded to salts and the other the S fibre (for small spikes) which responded to sugars (Hodgson *et al*. 1955; Hodgson and Roeder, 1956). Wolbarsht and Dethier (1958) were able to detect the spikes of the third neuron which terminated in a process at the base of the hair. It was designated M for mechanoreceptor. Evans and Mellon (1962) have now detected spikes from a fourth neuron which responds to water.

In the course of electrophysiological studies of chemoreceptor hairs it has been shown that when mixed stimuli are applied there is an interaction between activity in the L and S fibres (Hodgson, 1956a, 1957; Morita, 1959

Sturckow, 1959). Hodgson (1957) found that the presence of S impulses is accompanied by a decrease in L impulses and conversely the S spikes decrease when the L fibre is stimulated. My experiments show that the repellents block the reception of attractant and other stimuli. This assertion needs to be confirmed by electrophysiological methods.

The experiments described in Section 4.15 showed that mosquitoes with antennae painted with diethyl toluamide landed, walked around, and even probed on an arm also treated with the repellent but did not feed on blood. This can perhaps be explained in one of two ways. It may be that the piercing of the skin by the mosquito is induced by some chemical factor on the skin which was neutralized by the application of the repellent or, it may be due to the effect of repellent on the action of thermoreceptors or contact chemoreceptors which induce feeding on blood. The latter explanation would be more in conformity with the findings that repellents interfere with the reception of all kinds of stimuli affecting the total behaviour of mosquitoes.

The study on blood feeding when repellents were applied on parts of the mosquito revealed that of the four repellents dimethyl phthalate has the greatest effect on blood feeding behaviour when it is painted on the tarsal receptors and the smallest effect when it is painted on the receptors of the antennae. As is known, the olfactory receptors are located on the antennae and the contact chemoreceptors mostly on the tarsi of the mosquito. Dimethyl phthalate, which has the highest boiling point and hence the lowest vapour pressure, may, for this reason, have more effect than the other repellents through the tarsal chemoreceptors in the liquid phase but less than these through the olfactory receptors of the antennae where it has to act in the vapour phase which is at a lower concentration.

That repellents also acted as irritants was evident from the intense wriggling activity of the mosquito when repellents were applied on the proboscis and from the vigorous cleaning of repellent from the antennae with the tarsi of the fore legs. This evident awareness of the presence of an irritant chemical indicates the existence of receptors sensitive to it, perhaps those of the common chemical sense. It may well be that these are the only receptors not blocked by repellents.

In the vapour phase repellents were found to inhibit landing of mosquitoes. This was observed in experiments on blood feeding, sugar feeding, oviposition and air flow. In the liquid phase, however, the repellents showed more irritant and some toxic effects, and the mosquitoes showed considerable decrease in locomotor activity, in part on account of preoccupation with attempts at cleaning off the repellent.

Repellents have been defined as compounds which elicit an avoiding reaction (Dethier, 1956b). While the four materials studied may all do this, this is by no means their only effect and may not, indeed, be the most important one.

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